

Use of a mathematical model to investigate the influence of nitrogen consumption on pH during fermentation

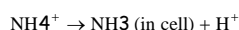
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This work deals with the use of a mathematical model to investigate the influence of the nitrogenous source on the pH during alcoholic fermentation in winemaking. If the influence of the assimilation of ammoniac by yeasts on pH is well developed in the literature, the results are more dubious concerning the assimilation of the amino acids. In order to discriminate various assumptions, a model of pH calculation developed by Akin et al. (2007) was used. So, fermentations in media with different nitrogenous source were carried out. The application of the model to fermentation medium whose nitrogen source was made up only of ammoniac gave very satisfactory results and confirms the assumption found in the literature that the assimilation of one mole of ammonia releases one mole of proton in the medium. The use of the model made it possible to invalidate two assumptions concerning the impact of the assimilation of the amino acids on the pH. The most probable assumption is that the assimilation of the molecules of amino acids charged positively led to the emission of protons in the extra cellular medium.

1. Introduction

Nitrogen compounds are important for the growth and the metabolism of yeast. It is quantitatively the second nutriment of the yeast, after the carbon element. During the alcoholic fermentation, in winemaking, nitrogen of grape must is assimilated by yeast cells for their growth. However, nitrogen is present under variable and complex forms: ammonium ions, amino acids, peptides and proteins. It is known that only ammonium ions and some amino acids are able to be metabolised by yeasts. The kinetic of the fermentation and aroma production are particularly influenced by the quantity and the nature of amino acids. So, the effect of nitrogen as precursors has been studied for long time (Cantarelli, 1957; Ough, 1964). More recently, the problem of yeast nitrogenous nutrition became a major research subject in oenology.

Various studies (Sigler and al., 1981; Kotyk, 1989) reported a pH decrease during alcoholic fermentation by *Saccharomyces cerevisiae* on medium where ammonium ions were the only nitrogenous source. The assimilation of ammonium ions by the yeast would be linked to the excretion of protons in the extra cellular medium. According to Won J.I and al. (1993) when one mole of ammonium is used, one mole of protons is excreted:



So, the decrease of pH would be directly proportional to the ammonium ions assimilated by yeasts.

Contrary to ammonium, the mechanisms of assimilation of amino acids by yeast seem more complex. Several authors (Grenson and al., 1970; Salmon, 1989) studied the type of exchange taking place between the intra cellular and extra cellular medium but no clear conclusion can be drawn from these works. The impact of the assimilation of amino acids by yeasts on the evolution of the pH is not clearly identified.

In previous studies (Akin et al., 2007), we proposed a mathematical model to calculate the pH of a grape must during the fermentation. This model has been validated on synthetic medium and natural grape musts. In this work, we propose to use this model to test different assumptions drawn on the assimilation of ammonium ions and amino acids.

Two fermentations with different nitrogenous source were performed.

2. Materials and methods

2.1. Micro organism

Saccharomyces cerevisiae QA-23 commercialised by Lallemand inc. was used as it is a classical yeast strain for white winemaking.

2.2. Fermentation media and operating conditions

Fermentation were carried out on synthetic media whose composition was close to grape must: glucose (200 g.L⁻¹), malic acid (6 g.L⁻¹), citric acid (6 g.L⁻¹), KH₂PO₄ (0.75 g.L⁻¹), K₂SO₄ (0.5 g.L⁻¹), MgSO₄ 7 H₂O (0.25 g.L⁻¹), CaCl₂ 2H₂O (0.16 g.L⁻¹), NaCl (0.2 g.L⁻¹), a mixture of oligo elements, vitamins and anaerobia factors. The nitrogen source differed with experiments: ammonium ions for an equivalent of 420 mg N.L⁻¹ for the medium MS_NH₄ and a mixture of amino acids for an equivalent of 305 mg N.L⁻¹ for the medium MS_AA. The pH of media was adjusted to 3.3 before autoclaving with NaOH solution (8N).

2.3. Compounds and pH measurement

The determination of cell concentration was done using an electronic analyser Beckman Coulter, model M3 according to the Coulter method "Electrical Sensing Zone". pH was measured on line with a precision of 0.05 unit of pH. Concentrations of ammonium, malic and lactic acids were measured by enzymatic kits (MicroDom). Citric, acetic and succinic acid, ethanol and glycerol were measured by HPLC method. Individual amino acid concentrations were determined by Biochrom 30 method.

2.4. pH calculation model

pH calculation model used in this study was developed by Akin et al. (2007). This model was based on thermodynamic equilibrium of electrolytic compounds in solution. The molality of hydrogen ions and thus the pH are determined by solving a non linear algebraic equations system consisted of mass balances, chemical equilibrium equations and electroneutrality principle.

3. Results and discussions

3.1. Fermentation of the MS_NH₄ medium

The fermentation on the MS_NH₄ medium was performed during 110h. Ammonium ions is the only nitrogen source. The evolution of the main compounds concentration is plotted on figure 1.

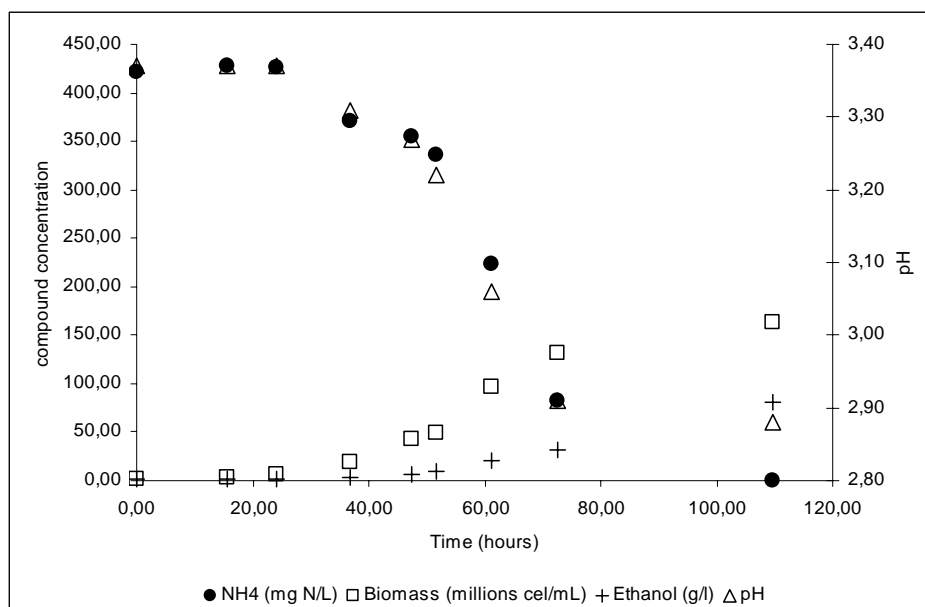


Figure 1. Evolution of biomass, ethanol, ammonium and pH during fermentation carried out on the MS_NH4 medium

During the first fermentation period (0 to 40 hours), the metabolic activity was slow: a slow consumption of ammonium ions (about 30 mgN.L⁻¹), a low production of biomass (3.10⁶ cells.mL⁻¹) and ethanol (4 g.L⁻¹). A little decrease of pH was observed, from 3.37 to 3.31. During the second period, between 40 and 110 hours, the metabolic activity increased. At the end of the fermentation, ethanol concentration was about 82 g.L⁻¹, biomass attained almost 160.10⁶ cells.mL⁻¹ and ammonium was entirely consumed. The decrease of pH was also emphasized since its final value was 2.90.

The measurement of organic acid concentrations indicates that very small quantities were produced: 0.16 g.L⁻¹ of succinic acid, 0.18 g.L⁻¹ for acetic acid. Citric and malic acids remained constant to 6 g.L⁻¹ during the whole fermentation.

3.2. pH Simulations

The pH calculated with the model (3.37) at the very beginning of the fermentation when the medium composition is perfectly known is in agreement with the experimental value. Some compounds contents were considered as constant during fermentation like for instance mineral cations. The others (ethanol, sugar, organic acids and ammonium) were considered to be variable and have been measured at different times for which pH values can be calculated by the model.

The model expresses the hypothesis formulated by Castrillo J.I et al. (1995) and Won J.I et al. (1993): when ammonium ions which are on the cation form (NH₄⁺) at the pH of the must are consumed by yeasts, one mole of proton is released to ensure the electroneutrality principle.

Results are shown in figure 2. They show a very good agreement between calculated and experimental pH values since the maximal deviation is less than the precision of the measure.

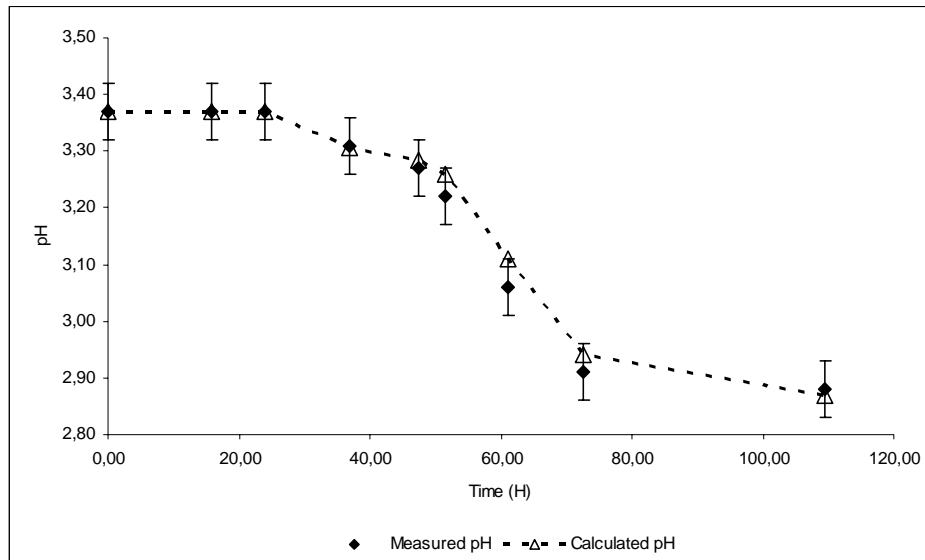


Figure 2. Comparison of experimental and simulated pH values in MS_NH4 medium (initial pH=3.37; T=20°C)

3.3. Fermentation of the medium MS_AA

In MS_AA medium, amino acids brought 305 mg of nitrogen by liter which could be assimilated by yeast. The fermentation has also been carried out and the evolutions of the major compounds concentrations are plotted on figure 3.

As for the fermentation on MS_NH4 medium, we can distinguish two periods. During the first period (0 to 20 hours), only 2 g.L⁻¹ of ethanol are produced, biomass attained only 3.10⁵ cells.mL⁻¹ and few amino acids were consumed (about 40 mgN.L⁻¹). pH kept constant to its initial value of 3.23. During the second period (20 to 110 hours), ethanol production increased to reach 94 g.L⁻¹ at the end of the experiment. Concentration of biomass rose to 200 millions cells per millilitre and amino acids were totally consumed. The measurement of organic acid concentrations revealed that only 0.3 g.L⁻¹ of succinic acid and 0.3 g.L⁻¹ of acetic acid were produced. Malic and citric acids remained constant to their initial concentration of 6 g.L⁻¹.

The pH value decreased from 3.23 to 3.13 after 50 hours of the fermentation which corresponds to the assimilation of amino acids. After this time, pH increased regularly to reach 3.25 at the end of the experiment. We have already shown (Akin et al., 2007) that the ethanol effect on the organic acids dissociation is responsible for this rise.

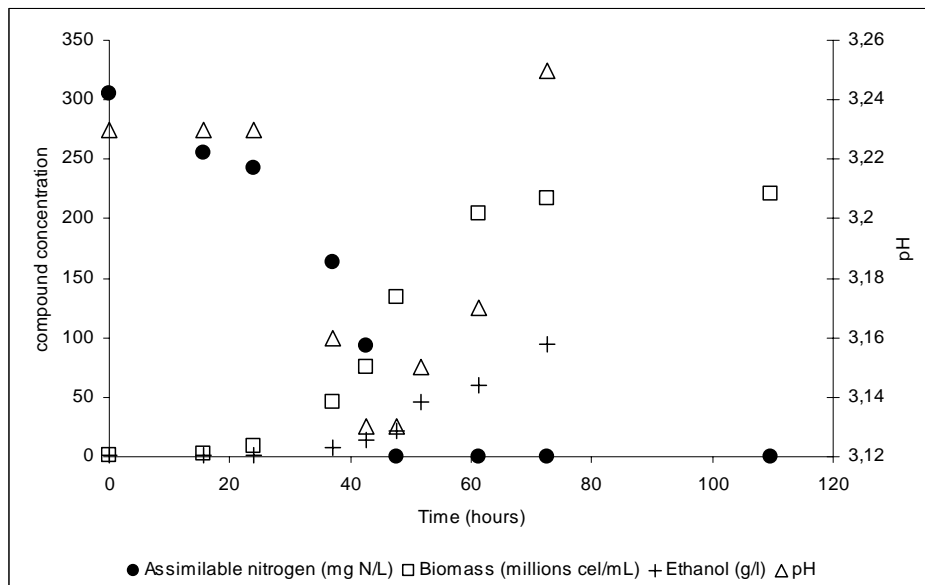


Figure 3. Evolution of biomass, ethanol, total amino acids and pH during fermentation carried out on MS_AA medium (initial pH=3.23; T=20°C)

3.4. pH simulations

As for MS_NH4 medium, the initial pH of MS_AA medium was perfectly predicted by the model. The knowledge of the medium composition allowed calculating an initial pH of 3.23. The complexity of the phenomena linked to the assimilation of amino acids by yeasts was underlined in the literature. The type of exchange which takes place when amino acids pass through cell membrane are not yet known with accuracy and certainty. To integrate the amino acids consumption in the model, three hypotheses were tested:

- Hypothesis 1: assimilation of amino acid does not influence the pH
- Hypothesis 2: consumption of one mole of amino acid causes the excretion of one mole of H⁺ like for the ammonium consumption
- Hypothesis 3: consumption 1 mole of amino acid charged positively only causes excretion of 1 mole of protons to respect electroneutrality principle.

Under these hypotheses, the pH evolution was simulated and compared to experimental data.

According to hypothesis 1, the decrease of pH observed between 0 and 50 h during the fermentation of medium MS_AA would not be caused by amino acids consumption. However, any other phenomenon could explain it since organic acids production was very low. So, the pH decrease seems to be necessarily linked to amino acids consumption and so hypothesis 1 is rejected.

pH were calculated under the hypothesis 2 (figure not showed). Minimum pH calculated was 3.03, that is lower than the experimental one (3.13). Thus, the assimilation of amino acids induces a decrease of pH but only some of them seems to cause the excretion of protons.

To test the third hypothesis, it is necessary to evaluate amino acids proportion according to their electronic charge. Each amino acid has an equilibrium constant (pK₁) for their

acid function (COOH) and an equilibrium constant (pK_2) for their amine (NH_2) function. Some of them also have a third equilibrium constant (pK_3) linked to their radical. With these values of pK , it is possible to determinate the proportion of each form of amino acids: cationic, anionic or neutral as a function of pH. For the initial pH of 3.23, table 1 gives the proportion of each amino acids of the must.

Table 1. Quantity of nitrogen brought by each amino acid

Amino acids	Aspartic acid	Thréonine	Sérine	Glutamic acid	Glutamine	Proline	Cystéine
% cation (2+)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
% cation (1+)	5,54	20,08	8,36	7,69	8,01	4,99	0,00
% neutral	76,52	79,92	91,64	84,27	91,99	95,01	0,00
% anion	17,94	0,00	0,00	8,05	0,00	0,00	0,00
%anion (2-)	0,00	0,00	0,00	0,00	0,00	0,00	0,00

Amino acids	Cystine	Méthionine	Isoleucine	Leucine	Tyrosine	Phénylalanine	Lysine
% cation (2+)	0,00	0,00	0,00	0,00	0,00	0,00	0,00
% cation (1+)	0,00	7,36	10,95	11,18	8,54	8,54	91,82
% neutral	0,00	92,64	89,05	88,82	91,46	91,46	0,00
% anion	0,00	0,00	0,00	0,00	0,00	0,00	0,00
%anion (2-)	0,00	0,00	0,00	0,00	0,00	0,00	8,18

Amino acids	Glycine	Alanine	Valine	Histidine	Tryptrophan	Arginine
% cation (2+)	0,00	0,00	0,00	0,00	0,00	0,00
% cation (1+)	11,65	11,41	12,63	3,74	14,52	91,99
% neutral	88,35	88,59	87,37	96,10	85,48	0,00
% anion	0,00	0,00	0,00	0,16	0,00	0,00
%anion (2-)	0,00	0,00	0,00	0,00	0,00	8,01

With these calculations and with the measure of individual concentration of amino acid, we were able to determine the total concentration of amino acids on cationic, anionic and neutral form during the fermentation (table 2).

In our medium, with amino acids repartition, there are 70% amino acids globally in neutral form, 27 % have 1 positive charge, 1,3 % have 2 positives charges and 1,3 % have only 1 negative charge.

In the model, to respect electroneutrality principle, each amino acid assimilated under the cationic form involves a proton excretion whereas each amino acid on anionic form involves neutralisation of one proton in the medium and amino acids on neutral form have no effect on proton concentration.

With these assumptions, simulation of pH values of the medium MS_AA was realised. Results are shown on figure 4. We noticed a good adequacy between the calculated and experimental pH. The maximal deviation was 0.04 point of pH that is less than the precision of the measure.

Table 2. Amino acids proportions according to their charge during fermentation

Time (hours)	Alpha amino nitrogen (mg N/L)	Positive amino acids (mg N/L) A+	Positive amino acids (mg N/L) A2+	Neutral amino acids (mg N/L) A±	Negative amino acids (mg N/L) A-	Negative amino acids (mg N/L) A2-
0	305,0	83,2	5,0	213,2	3,6	0,0
15,7	256,0	69,7	4,2	179,1	3,0	0,0
24	243,0	66,1	4,0	170,0	2,9	0,0
36,937	163,0	49,1	3,1	108,9	1,9	0,0
42,63	93,0	33,1	2,2	56,9	0,7	0,0
47,53	0,0	0,0	0,0	0,0	0,0	0,0
61,16	0,0	0,0	0,0	0,0	0,0	0,0
72,55	0,0	0,0	0,0	0,0	0,0	0,0
109,54	0,0	0,0	0,0	0,0	0,0	0,0

The simulation results were globally in agreement with the experimental data when hypothesis 3 was formulated. So, the global charge of amino acids would determine the pH evolution. However, additional experiments with different proportion of each amino acid are necessary to validate this hypothesis.

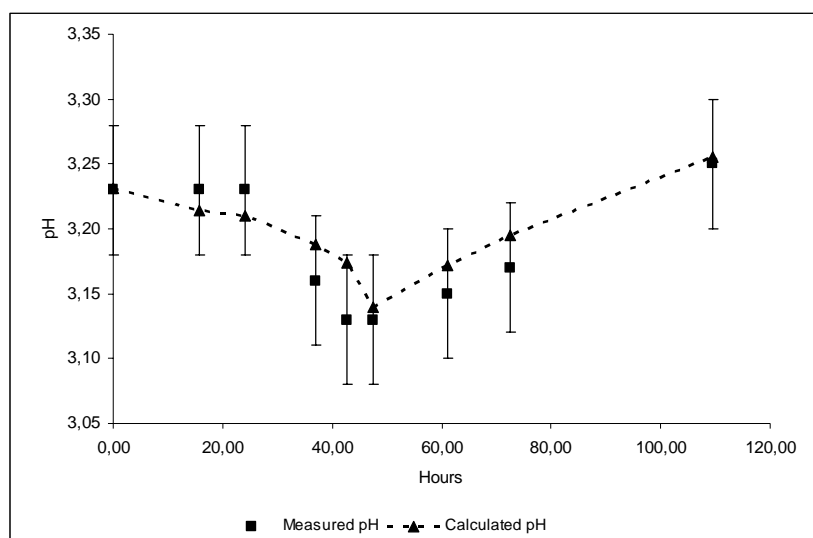


Figure 4. Comparison of experimental and calculated pH in MS_AA medium (Initial pH=3.23; T=20°C)

4. Conclusion

At the end of this study, it can be stated with certainty that the acidification of the medium during alcoholic fermentation is linked to the assimilation of the nitrogen source. On a medium where nitrogen was only brought by ammonium (420 mg N.L⁻¹),

the pH decreases down to 0.47 pH unit. With amino acids as nitrogen source (305 mg N.L⁻¹) the decrease of the pH was lower, only 0.1 unit of pH.

The model of pH allowed us to suggest that only the cationic form of amino acids was responsible for the pH decrease.

This assumption must be validated with additional fermentations realised on synthetic medium as well as on natural grape musts.

5. References

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