

Factors Affecting *Zymomonas mobilis* subsp. *francensis* Growth and Acetaldehyde Production

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ABSTRACT

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An experimental plan was designed to determine the incidence of factors encountered during cider production on *Zymomonas mobilis* subsp. *francensis* growth and acetaldehyde production. Different factor combinations of pH (3.50 to 4.10), SO₂ addition (0, 50, 100 and 200 mg/L), nitrogen source concentrations (0.5 and 5.0 g/L), polyphenol cider marc extract supplementation (0.25 and 1.00 g/L), temperature (12°C, 18.5°C and 25°C) and inoculation level (10² and 10⁵ CFU/mL) were tested over a 30-day period at regular time intervals in synthetic medium with ethanol. Viable cell counts and acetaldehyde production were correlated. Individually, and in decreasing significance, the following factors influenced acetaldehyde production: nitrogen source, SO₂ addition, inoculation level, temperature and pH. On the other hand, presence of polyphenol cider marc extract was not significant. A model was determined based on factor interactions. At high contamination levels (10⁵ CFU/mL), conditions leading to a high risk for spoilage were observed at pH values ranging from 3.75 to 4.10, at 0 to 50 mg/L total SO₂ and in the presence of 5 g/L nitrogen source (yeast extract) while temperature did not in fact appear to play a key role. At lower contamination levels (10² CFU/mL), the risk was drastically reduced. The only conditions leading to increased acetaldehyde levels were a high pH 4.1, no added SO₂ and high nitrogen source concentration (5 g/L yeast extract).

Key words: acetaldehyde, cider, experimental plan, metabolism, risk model, statistical analysis, synthetic medium, *Zymomonas mobilis*.

INTRODUCTION

In the cider industry, presence of the Gram-negative facultative anaerobic bacterium *Zymomonas mobilis* is regarded as unwanted since this bacterium is responsible for one of the major cider spoilages known as “cider-sickness” in England^{2,21} and “framboisé” in France^{9,13}. *Z. mobilis* spoils cider by forming high acetaldehyde concentrations (150–400 mg/L in general and up to 1000 mg/L, which are above the legal limits in France of 100 mg/L) causing off-flavours described as herbaceous, rotten lemon, raspberry-like or banana skin. Tremendous amounts of

gas (3 to 9 bars in bottles) and sometimes turbidity due to the combination of polyphenol compounds with acetaldehyde are also observed^{9,19}. In England, the subspecies *Z. mobilis* subsp. *pomaceae*² was found to be at the origin of this spoilage while “framboisé” is caused by the recently described subspecies, *Z. mobilis* subsp. *francensis*¹¹. Likewise, in the beer industry, growth of the third subspecies, *Z. mobilis* subsp. *mobilis*, was correlated with the production of large quantities of acetaldehyde and H₂S as well as a marked turbidity^{15,17}. Yet, this bacterium is not only involved in beverage spoilage. In traditional fermented products such as agave, palm and sugarcane saps, this bacterium plays a major role as part of the desirable fermentation flora for alcohol production²⁶.

All of these ecological niches contain high concentrations of simple sugars (glucose and fructose) favouring *Z. mobilis* growth²⁶. The most outstanding feature of this bacterium is the quantitative anaerobic fermentation of glucose to equimolar amounts ethanol and CO₂ with acetaldehyde as a precursor using the Entner-Doudoroff pathway at a rate twice as fast as yeast^{8,26}. On the other hand, only a few carbon sources are utilized by this bacterium. All *Z. mobilis* strains ferment glucose and fructose^{7,26} while only two of the three subspecies, *Z. mobilis* subsp. *mobilis*^{7,26} and *Z. mobilis* subsp. *francensis*¹¹, are able to metabolize sucrose within 48 h.

The pathway involved in ethanol production is identical to the one used by the yeast, *Saccharomyces cerevisiae*. Two essential enzymes are present; pyruvate decarboxylase (PDC), involved in converting pyruvate into acetaldehyde and CO₂, and alcohol dehydrogenase (ADH), further converting acetaldehyde into ethanol²⁰. Up to 98% of the substrate pyruvate is converted, *via* acetaldehyde, into ethanol and CO₂²². However, numerous studies have shown that acetaldehyde, a toxic precursor of ethanol, accumulates under aerobic conditions^{5,16,18,27,29} as well as during by-product formation (e.g., mannitol) from, in particular, the fermentation of fructose and/or sucrose^{28,29}. Both aerobic growth and the formation of certain by-products require reducing equivalents and are therefore in direct competition with the enzyme of the Entner-Doudoroff pathway, ADH, which also requires free NADH for ethanol production^{3,6,16,27,28}. In this case, biomass production is also low^{4,5,23}. Although acetaldehyde is known to inhibit growth and possibly have an inhibitory effect on fermentation, low levels have also been shown to stimulate growth²⁵.

In France, natural fermentations are still being carried out for cider production. This often involves partial fermentations in which key growth elements may remain in

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the final product including residual sugars (in the case of semi-sweet or sweet cider) and rich nitrogen sources (both yeast and apple juice origin). This provides an ideal niche for spoilage bacteria growth and is certainly one of the main reasons why multiple “framboisé” cases still occur each year in France. As the “framboisé” microbiological origin is now known to be *Z. mobilis* subsp. *francensis*^{9,11}, it is important to understand the factors influencing the development of this bacterium and the production of acetaldehyde in order to be able to propose effective preventive and curative treatments. A recent study, based on the physiological characterization in synthetic media of the novel subspecies type strain, *Z. mobilis* subsp. *francensis* AN0101, showed that this bacterium could easily grow in these cider conditions including temperatures as low as 4.0°C, pH values as low as 3.5, at 8.0% (v/v) ethanol, above the legal limit of SO₂ addition (200 total mg/L) and in the presence of up to 3.0 g/L cider marc polyphenol extract¹⁰.

In this study, an experimental plan was designed in order to identify a maximum number of factors and treatment conditions having a significant influence on *Z. mobilis* subsp. *francensis* growth and acetaldehyde production in a model medium with ethanol. The chosen factors corresponded to representative cider house conditions. The outcome of this study may lead to better preventive treatments allowing for the reduction of the alteration risk in French ciders.

MATERIALS AND METHODS

Bacterial strain

Zymomonas mobilis subsp. *francensis* AN0101 (LMG 22974^T and CIP108684^T), isolated from a “framboisé” cider from the Calvados area of France, was part of the ADRIA NORMANDIE bacterial collection. The characteristics and maintenance of this subspecies have been previously described⁹⁻¹².

Media and culture conditions

Pure cultures of *Zymomonas mobilis* subsp. *francensis* AN0101 were grown in zymomonads (Z) liquid medium (glucose 20 g/L and yeast extract 5 g/L) overnight without agitation or anaerobically (Oxoid, France) on Z agar (glucose 20 g/L, yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L and agar 15 g/L) for up to five days at 30°C⁹. Before inoculation into the test media, three consecutive precultures were performed.

All media for the experimental plan (see experimental design section below) contained 20 g/L glucose (Labogros, France) and 3% (v/v) absolute ethanol (added after samples were sterilized by autoclaving). Six different factors were studied and 34 different combinations of treatment variables were applied. The first factor, yeast extract (AES, France) representing the nitrogen source, was added from 0.5 g/L to 5 g/L final concentration to the base medium and autoclaved. Then, a sterile cider marc polyphenol extract was added at final concentrations of 0.25 to 1.0 g/L. Total polyphenol cider marc extract corresponded to 38.48% dry weight expressed in catechin equivalent and was supplied by Val de Vire Bioactives

(France). Next, pH adjustments from 3.50 to 4.10 were performed on the 150 mL samples. Finally, a filter-sterilized 10 ppm stock solution of SO₂ was added to obtain final concentrations of 0 to 200 mg/L total SO₂. Under acidic conditions, the active antimicrobial form of SO₂ will be predominant in solution, whereas when the pH becomes more basic, SO₃²⁻ will become predominant and can combine with other molecules present in the solution, such as polyphenols and acetaldehyde.

For inoculation of the samples, a stationary phase culture of strain AN0101, containing ~1.10⁸ CFU/mL, was diluted 10-fold once or four times in saline solution (TS, 10 g/L tryptone and 10 g/L NaCl, AES, France). Then, the diluted inoculum (1× or 4×) was added at 1% (v/v) into the given sample to obtain theoretical final cell concentrations of 1.10⁵ and 1.10² CFU/mL, respectively. Samples were incubated for 30 days at either 12°C or 25°C.

Experimental design

A fractional factorial experimental plan was designed using the Planor software (designed by A. Kobilinsky, INRA, France). This plan contained 6 factors, 2 of them with 4 levels: pH (3.50, 3.75, 3.85 and 4.10) and total added SO₂ (0, 50, 100 and 200 mg/L), and the others with 2 levels: temperature (12°C and 25°C), polyphenols (0.25 and 1.00 g/L), nitrogen source from yeast extract (0.5 and 5.0 g/L), and level of inoculum (1.10² and 1.10⁵ CFU/mL). The designed plan has a resolution of four, allowing for principal effects and second level interactions to be studied. A total of 34 treatments were tested in synthetically designed media, presenting conditions found in cider, over a 30 day period. The experimental central point was tested in triplicate (pH 3.80, 18.5°C, 0.88 g/L polyphenol extract, 2.75 g/L nitrogen source, 87.5 mg/L total SO₂ and ~1.10⁴ CFU/mL) and a control point was also added using optimal growth conditions (pH 4.1, 25°C, 0.25 g/L polyphenol extract, 1.10⁵ CFU/mL, 5.0 g/L nitrogen source, 0 mg/L SO₂ and 1.10⁵ CFU/mL). Sample preparation was randomized and analysis was carried out in the same order each time.

Enumeration of microorganisms

Sample analyses were performed at days 0, 1, 3, 7, 13, 20 and 30. For each sample, 1.0 mL was immediately used to enumerate viable cells in mass and/or by plating serial dilutions on Z agar with a Spiral System (Interscience, France). Negative controls were performed on Z agar without addition of sample to ensure medium sterility. Petri dishes were incubated at 30°C under anaerobic conditions for 3 to 5 days.

To ensure that no sample contamination occurred, total flora was plated on Plate Count Agar (PCA) (AES, France) and Petri dishes were incubated at 25°C under aerobic conditions for 72 h.

Analytical methods

Acetaldehyde was enzymatically analyzed for each sample using the Acetaldehyde UV-Method Kit (ref. 10668613035, r-biopharm, France) according to manufacturer's instructions. pH measurements were taken using a pH meter 763 Multi-calimatic (Knick, France). At day 30, alcohol content was analyzed using the methods described

in “Compendium of International Methods of Analysis of Wine and Must”¹.

Data analysis

Statistical analyses of the two studied continuous variables, cell numeration and acetaldehyde production, were performed using the SPAD program version 5.6.0 (SPAD SAS, Paris, France). The six factors; pH, temperature, polyphenol concentration, nitrogen source, SO₂ content, inoculation level as well as day of analysis, were used for statistical analysis. Obtained data were analyzed using the Desco procedure (SPAD S.A.S. software, Paris, France). This procedure, using the Fisher statistic, allows for continuous variables to be characterized and to determine factors having a significant effect on *Z. mobilis* growth and acetaldehyde production. Variance analysis of principal factors and interactions were performed with the Varg procedure (SPAD S.A.S. software, Paris, France) using the model (Variable = factor 1 + factor 2+...+factor 6 + factor 1 * factor 2 + + σ).

RESULTS AND DISCUSSION

Synthetic medium was chosen for this study to analyse the 6 chosen factors with different combinations of treatment variables rather than cider itself. Indeed, previous observations have clearly shown that *Z. mobilis* subsp. *francensis* growth appears to be cider-dependent (data not shown). Furthermore, use of synthetic medium eliminates the possibility of other unknown cider components influencing the results and facilitates factor concentration variations, such as nitrogen source and polyphenol concentrations.

Single factor and 2-way factor interaction analyses on *Z. mobilis* subsp. *francensis* growth

A total of 34 growth curves for *Z. mobilis* subsp. *francensis* corresponding to different combinations of pH, temperature, polyphenol cider marc extract concentrations, nitrogen source concentrations, total SO₂ addition and inoculation level, were generated for viable cell counts over time (data not shown). Factors were characterized individually and the generated probabilities were not significant (data not shown). Individual factor modality variance was also analyzed and similar results were obtained except for the absence of SO₂. In this case, increased growth was observed (data not shown). Presence of polyphenol cider marc extract (for the tested concentrations of 0.25 to 1.0 g/L) did not influence the outcome of growth and the same was observed for acetaldehyde production (see results discussed below). Preliminary experimental design studies showed that polyphenol concentrations of 1.5 to 4.0 g/L clearly inhibited *Z. mobilis* subsp. *francensis* growth at different pHs, total SO₂ additions and nitrogen concentrations (data not shown) so it was surprising to see no effect by this factor at slightly lower concentrations (1 g/L). Furthermore, in cider, the typical range of tannins is from slightly below 1, up to 3 g/L, with the majority closer to 1 g/L as the product becomes noticeably bitter at concentrations of 3 g/L and higher (P. Sanoner, personal communication).

Factor interactions on *Z. mobilis* subsp. *francensis* cell survival were then tested and average viable cell counts were determined for 4 different 2-way factor treatment combinations and are presented in Figs. 1A to D.

Effect of varying nitrogen source (yeast extract) and total SO₂ concentrations were most significant (Fig. 1A). However, their impact on viable cell counts was not as significant in comparison to acetaldehyde production (see results below). Temperature also had less of an effect on *Z. mobilis* subsp. *francensis* cell survival as observed at different nitrogen source and total SO₂ concentrations (Figs. 1B and C, respectively). In fact, at 12°C, average populations remained almost stable (1.10⁴ CFU/mL). The effect of pH and increasing total SO₂ concentration was also evaluated on viable cell counts (Fig. 1D). Clearly, *Z. mobilis* subsp. *francensis* grew at all pH values tested. Highest populations were observed between 3.75 and 4.10. This result was in accordance with previous physiological characterization data showing that this strain was able to grow at a pH as low as 3.5¹⁰ which is also characteristic of all strains of the species *Z. mobilis*^{11,26}.

Single factor variance analysis on acetaldehyde production by *Z. mobilis* subsp. *francensis*

A total of 34 curves for *Z. mobilis* subsp. *francensis* acetaldehyde production, corresponding to different combinations of pH, temperature, polyphenol cider marc extract concentrations, nitrogen source concentrations, total SO₂ addition and inoculation level, were generated (data not shown). The principal effects of each factor were evaluated in order to determine whether they significantly influenced acetaldehyde production. Significance probabilities are given in Table I. Clearly, nitrogen source concentration, quantity of total added SO₂, inoculation level (all had probability values of 1%) and to a lesser extent, incubation temperature and pH (both had probability values of 5%) influenced acetaldehyde production, as probability values were very highly significant and significant respectively.

Each factor was analysed using the Student t-test to determine which factor levels were significantly different. For temperature, both 25°C and 12°C were significantly different from the overall data average (both presented probabilities of 0.01). Concerning the temperature effect, the determined data average for 25°C (57.31) was significantly different and above the overall data average (41.86) which clearly indicated that higher acetaldehyde levels were observed at 25°C in comparison to 12°C. Indeed, at 25°C, levels up to 86 mg/L acetaldehyde were produced by *Z. mobilis* subsp. *francensis* while at 12°C, levels up to 46 mg/L were observed.

Increased nitrogen source concentrations (5 g/L yeast extract) also had a significant effect on acetaldehyde production (observed average significantly higher than data average). On the other hand, sample treatments with 0.5 g/L yeast extract as a nitrogen source appeared to have a negative effect and results confirmed this finding with acetaldehyde produced at levels only slightly above 100 mg/L. In a 2005 study by Bauduin *et al.*³, ciders inoculated with *Z. mobilis* subsp. *francensis* strain AN0102 were more susceptible to “framboisé” development when sup-

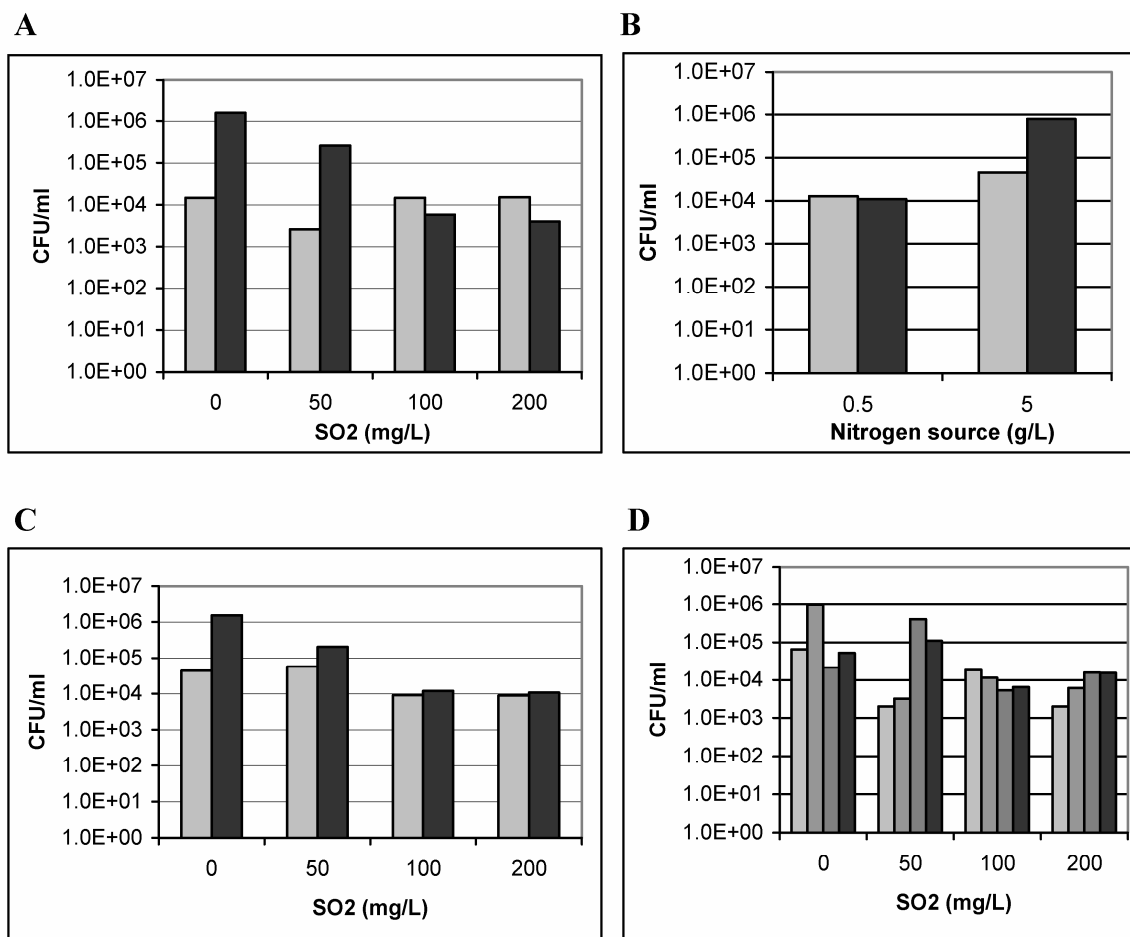


Fig. 1. Effects of factor interactions (A) nitrogen source (0.5 g/L, ■ ; 5.0 g/L, ■) and added SO₂, (B) temperature (12°C, ■ ; 25°C, ■) and nitrogen source, (C) temperature (12°C, ■ ; 25°C, ■) and added SO₂, (D) pH (3.50, ■ ; 3.75, ■ ; 3.85, ■ ; 4.10, ■) and added SO₂ on average *Z. mobilis* subsp. *francensis* cell survival.

Table I. Probability values for single factors which affect acetaldehyde production kinetics by *Z. mobilis* subsp. *francensis*.

Variable name	Number of degrees of liberty	Fisher	Test value	Probability ^a
Nitrogen source	229	20.26	4.40	0.000
SO ₂	227	8.81	4.18	0.000
Inoculation level	229	11.03	3.28	0.001
Temperature	229	5.51	2.33	0.020
pH	227	3.18	1.97	0.024
Polyphenols	229	2.36	1.53	0.126
Day	224	1.97	1.47	0.071

^a Critical probability ≤ 0.05 is considered significant (5%), very significant <0.01 (1%) and very highly significant <0.001 (1%).

plemented with 20 mg/L asparagine (assimilable nitrogen source). However, in synthetic medium containing a limited nitrogen source (50 mg/L yeast extract), asparagine did not increase growth at concentrations of up to 100 mg/L (data not shown).

Increased total SO₂ concentrations negatively affected *Z. mobilis* subsp. *francensis* acetaldehyde production, as lowest production levels were observed in the presence of 200 mg/L total SO₂ (SO₂ data average was below the overall average indicating a negative effect). Previous results showed that this strain was able to grow in the presence of 200 mg/L total SO₂ in Z medium (optimal growth medium at pH 5.8), although a longer lag phase was necessary.

However, at that time acetaldehyde production was not determined and no other factors were tested¹¹. Therefore, results presented in this study interestingly showed that this bacterium produced high levels of acetaldehyde (up to 67) at 50 mg/L, while acetaldehyde production was below the legal limit in the presence of 100 mg/L total SO₂. This result suggested that the addition of 50 mg/L total SO₂ had almost no impact (confirmed by the non-significant probability value) on the overall production of acetaldehyde in comparison to control conditions, under which 86 mg/L acetaldehyde was produced. The use of this molecule during cider-making is a common practice for controlling microbial populations^{14,24}. The above findings

Table II. Probability values for factor interactions which affect acetaldehyde production kinetics by *Z. mobilis* subsp. *francensis* (Temp = temperature; N = nitrogen source; Inoc = inoculation level).

Variable name	Sum of squares	Fisher	Critical probability ^a	Test value
Residual differences	1581410.000
Temp × N	38179.100	5.167	0.0240	2.26
N × SO ₂	139786.000	6.305	0.0004	3.35
SO ₂ × Inoc	83659.400	3.774	0.0113	2.28
Temp × SO ₂	83782.900	3.858	0.0101	2.32
N × Inoc	26295.500	3.536	0.0614	1.87
Temp × Inoc	1352.750	0.182	0.6702	-0.43
SO ₂	186484.000	8.412	0.0000	4.05
Nitrogen source	150173.000	20.322	0.0000	4.40
Inoculation level	74471.100	10.078	0.0017	3.13
Temperature	30203.900	4.087	0.0445	2.01
pH	51331.300	2.315	0.0762	1.43

^a Critical probability ≤ 0.05 is considered significant (5%), very significant <0.01 (1%) and very highly significant <0.001 (1%).

concerning limited acetaldehyde production in the presence of 100 or 200 mg/L total SO₂ (in conjunction with other factors) are not surprising in synthetic medium, in comparison to the elevated levels often observed in spoiled ciders even after addition of SO₂ (often between 50–85 mg/L total SO₂). In cider, it was previously shown that addition of 100 mg/L total SO₂ resulted in less than 15 mg/L free SO₂ after 24 h and levels were below 5 mg/L after 3 months in bottles (ARAC, personal communication). In ciders, multiple factors influence growth and acetaldehyde production and are still not well understood (in particular pH and nitrogen source). It is known that the antimicrobial activity of SO₂ is favoured at lower pHs due to the fact that this form is predominant (versus SO₃²⁻ at basic pH). Indeed, the same total SO₂ concentration range (0 to 200 mg/L) was tested at 3 different pH values. They were pH 3.7 (pH limiting “framboisé” spoilage), 4.1 (pH favouring “framboisé” cases) and 5.8 (optimal growth conditions). Growth of strain AN0101 was not influenced by SO₂ at pH 5.8 and 4.1 while at pH 3.7 and in the presence of increasing SO₂ concentrations, both growth and growth rate were slowed. Yet, this strain was still able to grow to near stationary phase levels after 72 h incubation (data not shown). This result also confirmed previous findings showing a strain-dependent resistance of up to 500 mg/L SO₂ for strains belonging to the subspecies *Z. mobilis* subsp. *pomaceae*²⁶. Recently, it was shown that cider inoculated with 10⁵ CFU/mL of *Z. mobilis* subsp. *francensis* strain AN0102 at pH 4.1, and in the presence of 200 mg/L SO₂, allowed for growth and acetaldehyde production³. In this case, SO₂ was not the predominant form.

Concerning inoculation level, results were in accordance with *Z. mobilis* subsp. *francensis* populations; more growth allowed for more acetaldehyde production. Variance analysis showed two distinct groups significantly different from the data average indicating that acetaldehyde production was higher in sample treatments with 10⁵ CFU/mL inoculum rather than 10² CFU/mL inoculum. Yet, up to 45 mg/L acetaldehyde was produced from 10² CFU/mL *Zymomonas* cultures but, in this case, no SO₂ was added and the other 4 factors were at near optimal levels (pH 4.1, 5 g/L yeast extract as nitrogen source, incubation temperature of 25°C and 1.0 g/L polyphenol cider marc extract).

For the chosen temperatures, both 12°C and 25°C were significant with higher acetaldehyde production observed at 25°C (86 mg/L) in comparison to 12°C (46 mg/L). Furthermore, at 12°C, populations only reached 10⁵ CFU/mL versus 10⁷ CFU/mL at 25°C. Although significant, these results, applied to cider conditions, tend to show that growth and high acetaldehyde levels can be reached in time.

The range of pH tested had the least effect on acetaldehyde production amongst the significant factors. A characterisation of variable modalities for pH showed that pH 4.1 and 3.5 were both significant (above and below the data average, respectively). For this reason, the higher the pH, the higher the probability of acetaldehyde production. However, up to 10 mg/L acetaldehyde was produced by this strain at pH 3.5 and in the presence of 5 g/L yeast extract as nitrogen source (optimal growth concentration), 1.0 g/L polyphenol cider marc extract, no added SO₂ (absence of an antimicrobial agent), an inoculum of 1.10⁵ CFU/ml and at 12°C (this temperature was shown to allow for stationary phase growth of the bacterium although a longer lag phase was necessary¹⁰).

Concerning polyphenol concentrations, their overall effect was observed to be non-significant (probability values > 0.05). In comparison to previous physiological test results on this strain, it is surprising to find that polyphenol concentration did not influence acetaldehyde production to a larger extent, as survival of this bacterium in the presence of increased polyphenol concentrations was observed to dramatically decrease over time in synthetic medium¹⁰.

Analysis of two-way factor interactions on acetaldehyde production by *Z. mobilis* subsp. *francensis*

Factor interactions on acetaldehyde production by *Z. mobilis* subsp. *francensis* were tested using the model described in Materials and Methods and results are given in Table II.

Only significant results were used in the final model. Analysis of variance showed that *Z. mobilis* subsp. *francensis* acetaldehyde production kinetics was affected by four of the above mentioned factors; temperature, yeast extract nitrogen source concentration, quantity of added total SO₂ and inoculation level. In general, decreased tem-

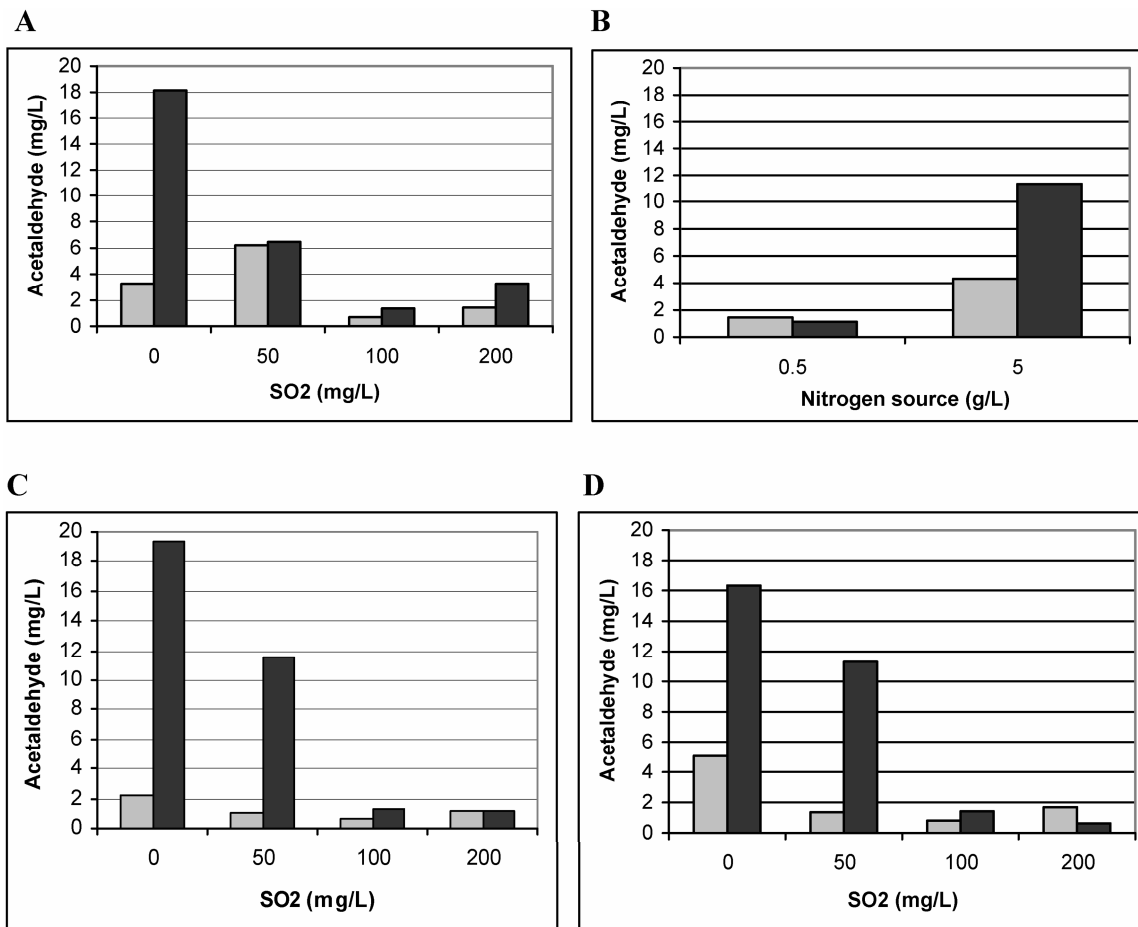


Fig. 2. Effects of factor interactions (A) temperature (12°C, ■ ; 25°C, ■) and added SO₂, (B) temperature (12°C, ■ ; 25°C, ■) and nitrogen source, (C) nitrogen source (0.5 g/L, ■ ; 5.0 g/L, ■) and added SO₂ (D) inoculation level (10², ■ ; 10⁵, ■) and added SO₂, on average acetaldehyde production by *Z. mobilis* subsp. *francensis*.

perature, nitrogen source and inoculation level, as well as increased SO₂ concentrations, tended to prevent elevated levels of acetaldehyde production by this strain. These results were also correlated to *Z. mobilis* subsp. *francensis* growth; high growth levels were observed with increased acetaldehyde production (data not shown). Interaction effects based on average acetaldehyde production values using significant treatment combinations are illustrated in Figs. 2A to D.

Clearly, average acetaldehyde production values were higher at 25°C than 12°C in the absence of SO₂. However, temperature no longer appeared to play a major role at concentrations of 50, 100 and 200 mg/L total SO₂ (Fig. 2A). Concerning nitrogen source, at low levels, average values for acetaldehyde production were low at both 12 and 25°C. On the contrary, increased nitrogen source concentrations clearly increased average acetaldehyde production values at 25°C (at 12°C, values were similar to those obtained with low nitrogen source concentrations) (Fig. 2B). Analysis of nitrogen source and SO₂ concentrations on average acetaldehyde production values showed that high nitrogen source concentrations, in the absence or presence of 50 mg/L SO₂, allowed for increased acetaldehyde production while limited amounts were produced at both 100 and 200 mg/L SO₂ (Fig. 2C). Finally, high inoc-

ulation levels in the presence of up to 50 mg/L SO₂ enabled this strain to produce elevated amounts of acetaldehyde (Fig. 2D).

Risk model for *Z. mobilis* subsp. *francensis* growth and acetaldehyde production

In synthetic medium, mimicking cider fabrication conditions, treatments proven to be effective in preventing acetaldehyde accumulation included various combinations of pH, SO₂, nitrogen source and inoculation level. A model was deduced from the above findings and is presented in Fig. 3.

High risk conditions for acetaldehyde production with an inoculation level of 10⁵ CFU/mL included the following treatment combinations: pH values ranging from 3.75 to 4.10, 0 to 50 mg/L SO₂ and 5 g/L yeast extract as nitrogen source (equivalent to 500 mg/L nitrogen content). Clearly, amongst all factor interactions, the strongest relationship in regards to *Z. mobilis* subsp. *francensis* growth and acetaldehyde production was observed between nitrogen source concentration and SO₂ addition. From a statistical point of view, temperature did not in fact appear to play a key role in elevated acetaldehyde level production; however, under specific conditions, namely 0 mg/L total SO₂ and 5 mg/L nitrogen source (Figure 2A and B, re-

Inoculation with 10 ⁵ CFU/mL :					
pH \ SO ₂	0	50	100	200	Temp. °C:
3.50	MEDIUM RISK		LOW RISK		12, 25
3.75	HIGH RISK		MEDIUM	LOW RISK	12, 25
3.85			RISK		12, 25
4.10	HIGH RISK		LOW RISK		12, 25
Nitrogen:	5	5	5.0, 0.5	5.0, 0.5	
Inoculation with 10 ² CFU/mL :					
pH \ SO ₂	0	50	100	200	Temp. °C:
3.50	MEDIUM RISK		LOW RISK		12, 25
3.75					12, 25
3.85	HIGH RISK		LOW RISK		12, 25
4.10					12, 25
Nitrogen:	5	5	5.0, 0.5	5.0, 0.5	

Fig. 3. Predicted model for the assessment of risk factors leading to acetaldehyde production by *Z. mobilis* subsp. *francensis*.

spectively), a temperature of 25°C allowed for significant acetaldehyde production in comparison to 12°C. When the inoculation level was lowered to 10² CFU/mL, the only high risk combination was a pH of 4.1 in the absence of SO₂ and an elevated nitrogen source concentration (5 g/L yeast extract).

The results obtained during the experimental plan provided information on the impact of different factors in regards to acetaldehyde production by *Z. mobilis* subsp. *francensis*. Increased levels of acetaldehyde in French ciders, characterized as “framboisé” and considered to be spoiled from organoleptic and legislative points of view, are known to be caused by this subspecies. It is therefore essential to determine factors that inhibit *Z. mobilis* subsp. *francensis* acetaldehyde production in this product. Survival of this bacterium in cider depends of a number of extrinsic (temperature, limited oxygen content...) and intrinsic (pH, carbon and nitrogen source availability...) factors related to growth requirements^{11,26} of which residual sugars certainly play a very key role. Several steps during cider processing are at risk for *Z. mobilis* contamination (for example, apple pulp, press,...). Yet, others can also be considered to cause bacterial stress such as low pH environments (juice from some acidic cider apple varieties can have a pH near 3.0), naturally present organic acids such as malic or citric acid and the presence of antimicrobial agents or preservatives such as SO₂. The results presented during this study showed that, individually, SO₂ addition, nitrogen source concentration, inoculation level, temperature and pH all influenced *Z. mobilis* subsp. *francensis* growth and acetaldehyde production in synthetic medium. In relation to cider, these factors would have a serious impact on the development of the “framboisé” spoilage and the characteristics often observed (development of off-flavours and odours due to acetaldehyde or abundance of gas).

From these results based on factor interactions, the risk of “framboisé” development in cider can certainly be lowered by the addition of SO₂ (especially in the case of prevention or at low contamination levels), removing excess nitrogen sources (for example by centrifugation of yeast and/or bacteria, re-inoculation of fermentative yeast) and by acidification of the product; these adjustments should be adapted to the contamination level. On the other hand, in the industry and according to the means of the cider house, fermentation temperature control will probably not be a factor that can be adjusted to lower contamination risk. Furthermore, it has been shown that *Z. mobilis* subsp. *francensis* grows at temperatures as low as 4°C to stationary phase, so in the case of contaminated cider, it is unlikely that the temperature alone will provide the necessary microbial control¹⁰. Overall, these results should help provide the cider industry with new methodologies to prevent this spoilage in French ciders.

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REFERENCES

1. Recueil des méthodes internationales d'analyse des vins et des moûts OIV Paris. 1990.
2. Barker, B. T. P. and Hillier, V. F., Cider sickness. *Annu. Rep. Agric. Hort. Res. Stn. Long Ashton Bristol*. 1912, 174-181.
3. Bauduin, R., Le Queré, J. M., Coton, E. and Primault, J.,

- Factors leading to the expression of “framboisé” in French ciders. *LWT-Food Sci. Technol.*, 2006, **39**(9), 966-971.
4. Belaich, J. P. and Senez, J. C., Influence of aeration and pantothenate on growth yields of *Zymomonas mobilis*. *J. Bacteriol.*, 1965, **89**, 1195-1200.
 5. Bringer, S., R. K. Finn, and H. Sahn. Effect of oxygen on the metabolism of *Zymomonas mobilis*. *Arch. Microbiol.*, 1984, **139**, 376-381.
 6. Bringer-Meyer, S. and Sahn, H., Acetoin and phenylacetylcarbinol formation by the pyruvate decarboxylases of *Zymomonas mobilis* and *Saccharomyces carlsbergensis*. *Biocatalysis*, 1988, **1**, 321-331.
 7. Carr, J. G., The Genus *Zymomonas*. The Prokaryotes. A Handbook on Habitats, Isolation and Identification of Bacteria, vol. II, H. S. Mortimer, P. Starr, G. Hans, A. Trueper, A. Balows and H. G. Schlegel, Eds., Springer-Verlag: NY, 1986, pp. 1349-1354.
 8. Conway, T., The Entner-Doudoroff pathway: history, physiology and molecular biology. *FEMS Microbiol. Rev.* 1992, **9**, 1-27.
 9. Coton, E. and Coton M., Microbiological origin of “Framboisé” in French ciders. *J. Inst. Brew.*, 2003, **109**, 299-304.
 10. Coton, M., Laplace, J. M., Auffray, Y. and Coton, E., “Framboisé” spoilage in French ciders: *Zymomonas mobilis* implication and characterization. *LWT-Food Sci. Technol.*, 2006, **39**(9), 972-979.
 11. Coton, M., Laplace, J. M., Auffray, Y. and Coton, E., Polyphasic study of *Zymomonas mobilis* strains revealing the existence of a novel subspecies *Z. mobilis* subsp. *francensis* subsp. nov. isolated from French cider. *Int. J. Syst. Evol. Microbiol.*, 2006, **56**(1), 121-125.
 12. Coton, M., Laplace, J. M. and Coton, E., *Zymomonas mobilis* subspecies identification by amplified ribosomal DNA restriction analysis (ARDRA). *Lett. Appl. Microbiol.*, 2006, **40**, 152-157.
 13. Drilleau, J. F., Le framboisé dans les cidres. *BIOS*, 1977, **12**, 37-44.
 14. Herrero, M., Garcia, L. A. and Diaz, M., The effect of SO₂ on the production of ethanol, acetaldehyde, organic acids, and flavor volatiles during industrial cider fermentation. *J. Agric. Food Chem.*, 2003, **51**, 3455-9.
 15. Ingledew, W. M., Effect of bacterial contaminants on beer. A review. *J. Am. Soc. Brew. Chem.*, 1979, **37**, 145-150.
 16. Ishikawa, H., Nobayashi, H. and Tanaka, H., Mechanism of fermentation performance by *Zymomonas mobilis* under oxygen supply in batch culture. *J. Ferm. Bioeng.*, 1990, **70**, 34-40.
 17. Jespersen, L., and Jakobsen, M., Specific spoilage organisms in breweries and laboratory media for their detection. *Int. J. Food Microbiol.*, 1996, **33**, 139-55.
 18. Kalnenieks, U., Galinina, N., Toma, M. M. and Poole R. K., Cyanide inhibits respiration yet stimulates aerobic growth of *Zymomonas mobilis*. *Microbiol.*, 2000, **146**(6), 1259-1266.
 19. Lea, A. G., Cidermaking. In: Fermented Beverage Production. A. G. H. Lea and J.R. Piggott, Eds., Chapman and Hall: London, U. K., 1995, pp. 66-96.
 20. Mackenzie, K. F., Eddy, C. K. and Ingram, L. O., Modulation of alcohol dehydrogenase isoenzyme levels in *Zymomonas mobilis* by iron and zinc. *J. Bacteriol.* 1996, **171**, 1063-1067.
 21. Millis, N. F., A study of the cider-sickness bacillus-a new variety of *Zymomonas anaerobia*. *J. Gen. Microbiol.*, 1956, **15**, 521-528.
 22. Neveling, U., Klasen, R., Bringer-Meyer, S. and Sahn H., Purification of the pyruvate dehydrogenase multienzyme complex of *Zymomonas mobilis* and identification and sequence analysis of the corresponding genes. *J. Bacteriol.*, 1998, **180**, 1540-1548.
 23. Pankova, L. M., Shvinka, Y. E., Beker, M. E. and Slava, E. E., Effect of aeration on *Zymomonas mobilis* metabolism. *Mikrobiologiya*, 1985, **54**, 141-145.
 24. Romano, P. and Suzzi, G., Sulphur dioxide and wine microorganisms. In: Wine Microbiology and Biotechnology. G. H. Fleet, Ed., Harwood Academic Publishers: Amsterdam, 1993, pp. 373-393.
 25. Stanley, G. A., Hogley, T. J. and Pamment N. B., Effect of acetaldehyde on *Saccharomyces cerevisiae* and *Zymomonas mobilis* subjected to environmental shocks. *Biotechnol. Bioeng.*, 1997, **53**, 71-78.
 26. Swings, J. and DeLey, J., The biology of *Zymomonas*. *Bacteriol. Rev.*, 1977, **41**, 1-46.
 27. Viikari, L., Carbohydrate metabolism in *Zymomonas mobilis*. *Crit. Rev. Biotechnol.*, 1988, **7**, 237-261.
 28. Viikari, L., and Korhola, M., Fructose metabolism in *Zymomonas mobilis*. *Appl. Microbiol. Biotechnol.*, 1986, **24**, 471-476.
 29. Wecker, M. S. A., The production of acetaldehyde using *Zymomonas mobilis*. PhD thesis, 1987, Cornell University.

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